Estramustine phosphate but not estramustine inhibits the interaction of microtubule associated protein 2 (MAP2) with actin filaments

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Abstract The effect of estramustine and estramustine phosphate (EP) on the interaction of microtubule associated protein 2 (MAP2) with actin has been examined. We show that (a) neither estramustine nor EP influences actin polymerisation (b) EP, but not estramustine, reduces the amount of MAP2 which cosediments with F-actin in a dose-dependent manner and (c) EP decreases the MAP2-induced crosslinking of F-actin into gelled networks. The data suggest, that unlike estramustine, EP interacts with MAP2 and modifies its interaction not only with microtubules but also with actin filaments.

Key words: Microtubule-associated protein; High-molecular weight MAP; Cytoskeleton; Microfilament

1. Introduction

Estramustine, an antineoplastic drug used in the treatment of prostatic carcinoma, is clinically administered as the phosphorylated analogue estramustine phosphate (EP) which is subsequently dephosphorylated in the cellular compartment [1]. Evidence has been presented both in vivo and in vitro suggesting that estramustine acts as an antimitotic drug by binding either to tubulin [2,3] or to MAPs [4,5]. In vivo estramustine causes an abnormal pattern of microtubule complexes and an alteration of the assembly/disassembly equilibrium only at high drug concentrations [6] or on long exposure [5], while in vitro it has been suggested to affect MAP-binding [4,5] or alternatively to show no effect on microtubule polymerisation [7]. On the other hand, EP interacts with MAPs and possesses antimicrotubule activity both in vitro [8] and in vivo [9,10].

MAP2 has been shown to interact with both microtubules and actin filaments [11–13] and to crosslink actin filaments, consequently possessing two or more actin binding sites [11–13]. At least one of the actin binding sites resides in the C-terminal domain containing the microtubule binding site [14]. This domain is known to be multiply phosphorylated [15,16] and although the phosphorylation does not affect MAP2:actin interaction it reduces the extension of actin filament networks [11], and the association of the protein with microtubules [17].

MAP2 has also been shown to possess five to six EP-binding sites residing in the positively charged tubulin binding domain [18]. It is therefore possible that the introduction of additional charge from the phosphate moiety of EP could weaken the MAP:tubulin interaction and cause microtubule depolymerisation, an effect similar to that described for phosphorylation [15,17].

In view of the potential interaction of estramustine with tubulin and of an additional interaction of EP with MAPs, it is difficult to understand fully the mechanism of action of EP. We studied the effect of estramustine and EP on MAP interaction with actin with the aim of elucidating the differences in the mode of action of these two compounds.

2. Materials and methods

The following buffer was used for all experiments described below: MES buffer (MES, 0.1 M; EGTA, 2.5 mM; $MgCl_2$, 0.5 mM; EDTA; 0.1 mM; pH 6.4 with NaOH). All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and all chemicals were of Analar grade.

2.1. Protein purification and composition

Twice cycled calf brain microtubule protein was prepared according to Pedrotti and Islam [19]. Rabbit skeletal muscle actin and MAP2 were purified as described by Pedrotti et al. [20].

Protein concentration was determined using Bio-Rad (Rockville Centre, NY) protein reagent and bovine serum albumin used as standard. SDS-PAGE was performed using the 'Phast System' (Pharmacia, Piscataway, NJ) and gels were stained with Coomassie blue R-250 [19].

2.2. Assay procedures

G-Actin (2 mg/ml) was polymerised in MES buffer containing 2 mM $MgCl_2$ at 37°C for 25 min. After polymerisation F-actin was diluted to 0.65 mg/ml and incubated at 37°C for 15 min with or without a final concentration of 0.15 mg/ml of MAP2 in either the presence or absence of estramustine or EP. At the end of the incubation period aliquots were removed and assayed either for actin binding or actin crosslinking studies.

For the actin binding studies the samples were centrifuged at 37°C for 25 min at $100\,000 \times g$ in a TL-100 centrifuge (Beckman Instruments Ltd., Toronto, Ontario). The protein in the pellet was fractionated by SDS-PAGE and stained with Coomassie blue R-250 [20]. For crosslinking studies the absorbance at 340 nm was determined using a Titertek Multiskan MCC/340 microtiter plate reader. Aliquots were also sedimented by centrifugation at $30\,000 \times g$ in a TL-100 centrifuge as described by Pedrotti et al. [20].

3. Results

The effect of estramustine and EP on MAP2:F-actin interaction was examined using the sedimentation assay. MAP2 was therefore incubated either in the absence of F-actin (con-

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Abbreviations: ATP, adenosine 5′-triphosphate; MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino)ethanesulphonic acid; Tris, Trizma base; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PMSF, phenylmethane sulphonyl fluoride; EP, estramustine phosphate

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trol) or with F-actin either in the absence of drug or in the presence of 100 μM estramustine or 400 μM EP. At the end of the incubation time, the samples were centrifuged at high speed (100 000×*g* for 25 min) and the pellets analysed by SDS-PAGE. Significant amounts of MAP2 were observed to co-sediment with F-actin (Fig. 1, lane 3), but not in the control sample in the absence of F-actin (not shown). Similarly, MAP2 was also observed to co-sediment with F-actin incubated in the presence of estramustine (Fig. 1, lane 2), suggesting that estramustine does not affect MAP2:actin interaction. By contrast, MAP2 failed to sediment when incubated with actin filaments in the presence of EP (Fig. 1, lane 1) indicating that the phosphorylated analogue affects the MAP2:actin interaction.

The effect of EP on F-actin:MAP2 interaction was therefore further examined at different concentrations of EP using the sedimentation assay. As shown in Fig. 2A, the amount of MAP2 which co-sedimented with F-actin, while unaffected at low concentrations of EP, was gradually reduced as the EP concentration increased. The dose-dependent inhibition by EP exhibits an IC_{50}, i.e. the amount which inhibits the interaction by 50%, of about 180 μM with complete inhibition observed at about 240 μM EP. This inhibition cannot be attributed to an effect of EP on actin as similar amounts of F-actin were pelleted when G-actin was polymerised in either the absence or presence of either estramustine or EP (Fig. 2B).

As MAP2 is also an actin crosslinking protein, we have additionally investigated the effect of EP on MAP2-induced actin crosslinking. Formation of filament networks leads to light scattering and can be monitored by the change in absorbance at 340 nm (Fig. 3). The absorbance of either F-actin or MAP2 alone was low and did not change during incubation at 30°C, and represented the background, while the co-incubation of F-actin with MAP2 (molar ratio MAP2:actin of

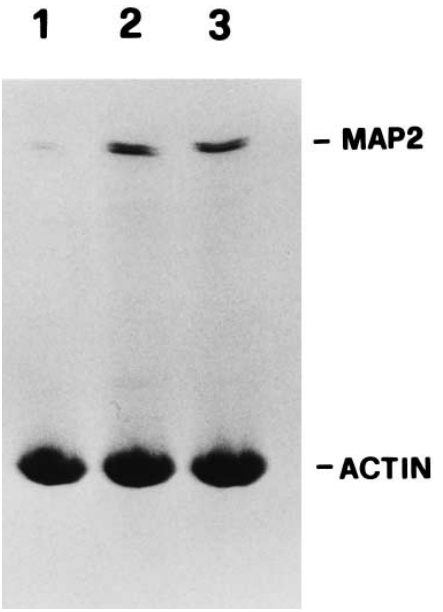

Fig. 1. Effect of estramustine and EP on MAP2:actin interaction. F-Actin was incubated with MAP2 either in the presence of 400 μm EP (lane 1) or 100 μm estramustine (lane 2) or in the absence of drug (lane 3). After centrifugation the pellets were resuspended and fractionated by SDS-PAGE on 4–15% acrylamide gradient gels. The gels were stained with Coomassie blue; the positions of MAP2 and actin are indicated.

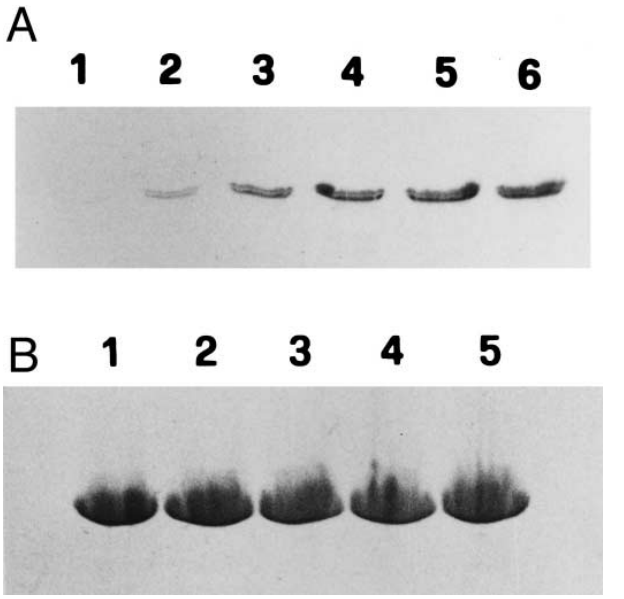

Fig. 2. Effect of increasing concentrations of EP on MAP2:actin interaction (A) and actin polymerisation (B). (A) MAP2 was incubated with F-actin either in the presence of increasing concentration of EP or in the absence of drug. After centrifugation the pellets were analysed by SDS-PAGE (see Fig. 1) and the recovery of MAP2 is shown. Lanes: 1, 300 μm EP; 2, 240 μm EP; 3, 180 μm EP; 4, 120 μm EP; 5, 60 μm EP; 6, control in the absence of EP. (B) G-Actin was polymerised at 30°C for 25 min in the presence of different concentrations of EP. At the end of the incubation period F-actin was sedimented by centrifugation and the pellets analysed by SDS-PAGE and the recovery of actin is shown. Lanes: 1, 400 μm EP; 2, 300 μm EP; 3, 200 μm EP; 4, 100 μm EP; 5, control in the absence of EP.

1:20 used) for 15 min at 30°C resulted in an increase in absorbance at 340 nm (Fig. 3a), and represented the signal. F-Actin was therefore incubated for 15 min at 30°C with various concentrations of EP in either the presence or absence of MAP2 and the absorbance change at 340 nm was determined at end of the incubation period. The absorbance at 340 nm, corrected for the specific absorbance of EP and MAP2, was plotted vs. the initial EP concentration. In the presence of increasing EP concentrations, the maximal signal observed with MAP2 and F-actin was gradually reduced until it achieved background levels in the presence of 180 μM EP (Fig. 3b). Aliquots were also removed and centrifuged at low speed (30 000×*g* for 40 min) through a 30% sucrose cushion and pelleted protein was fractionated by SDS-PAGE. As shown in Fig. 4, while only a small amount of actin was recovered in the pellet when F-actin alone was incubated, the amount of pelleted actin was substantially increased after addition of MAP2, and MAP2 was co-sedimented with the gelled actin (Fig. 4, lanes 1,3). On the other hand, the amount of actin which pelleted in the presence of 180 μM EP was similar to that of F-actin incubated in the absence of MAP2. Moreover, unlike the cross-linked samples only trace amounts of MAP2 could be detected in the pellets (Fig. 4, lane 2), confirming the complete disruption of microfilament networks in these conditions.

4. Discussion

EP interacts with MAP2 at the C-terminal region contain-

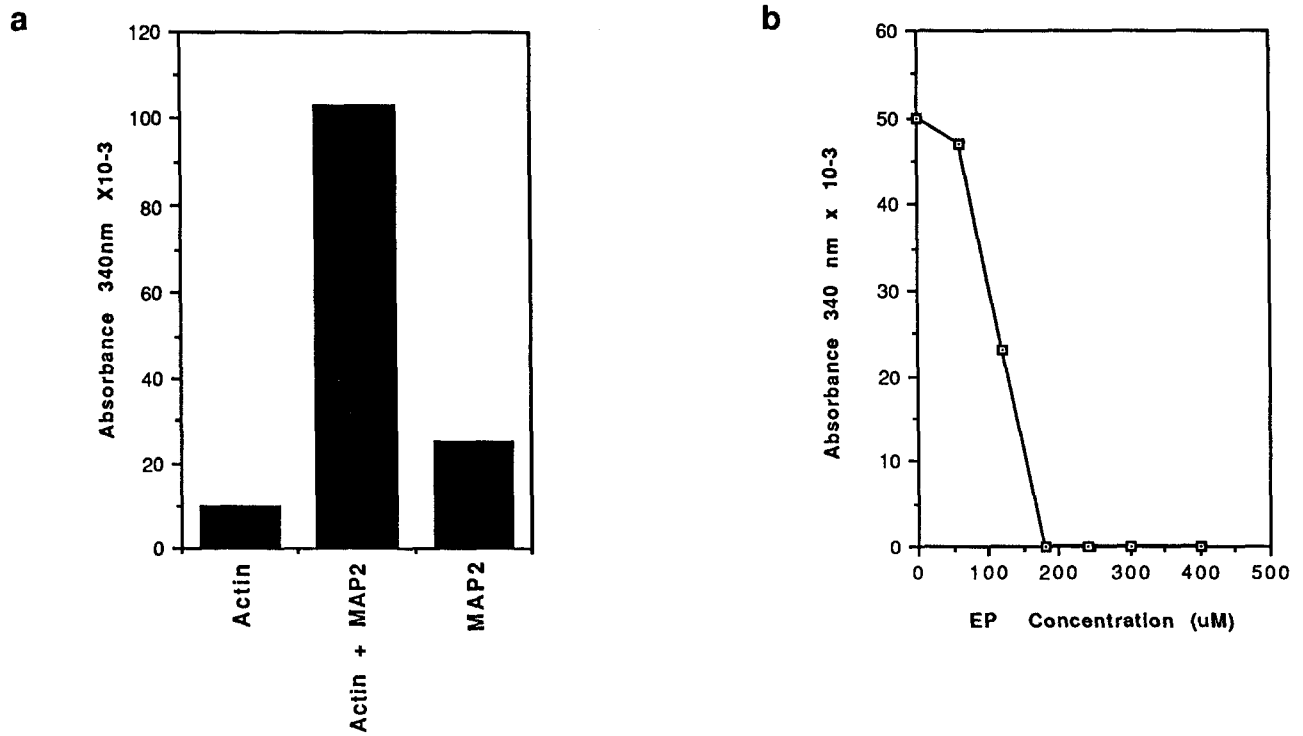

Fig. 3. MAP2-induced crosslinking of F-actin (a) and the effect of EP on crosslinking (b). (a) The absorbance of F-actin or MAP2 or MAP2 and F-actin was determined at 340 nm using a microtiter plate reader. A change in the absorbance was observed upon crosslinking of F-actin when incubated for 15 min in the presence of MAP2. (b) The effect of EP on MAP2-induced crosslinking was determined and plotted as a function of EP concentration.

ing the microtubule binding domain to inhibit the association of MAP2 with microtubules and to induce microtubule depolymerization [8,9]. However, MAP2 also binds and crosslinks actin filaments but, to the best of our knowledge, the effect of EP on MAP2:actin interaction has not been examined and this paper represents the first such study.

Our data suggest that EP affects the binding of MAP2 to actin filaments and induces the disruption of microfilament networks. A drug concentration of 240 μM is necessary to inhibit MAP2:actin binding while a concentration of 180 μM is sufficient to inhibit completely the crosslinking activity. This difference is probably due to the number of multiple interactions required to form a gelled network when compared with a single site for binding. By contrast, estramustine does not inhibit MAP2:actin binding (no effect was observed even at 200–400 μM estramustine, although at these concentrations the drug tended to precipitate), suggesting that it should not disrupt the microfilament networks.

The effect of EP is exerted most probably only through binding to the MAP2 molecule as it has no detectable effect on G-actin polymerisation. The relatively high drug concentrations necessary to induce the described effects are in agreement with the observation that MAP2 can bind 5–6 mol/mol EP [18]. The drug concentrations required to disrupt MAP2:actin interactions when compared with MAP2:microtubule interactions are somewhat lower in the case of the former interaction.

Considering that (a) EP binds to the MAP2 C-terminal microtubule binding domain [7,8,18], (b) MAP2:tubulin interaction involves charged domains of the two proteins [21], (c) the effect of monovalent and divalent cations suggests a similar electrostatic screening interaction for the MAP2:tubulin to that for the MAP2:actin complexes [13], (d) MAP2 microtubule binding domain also binds to G-actin [14], and (e) MAP2 binding sites on tubulin and actin molecules display a rather high degree of homology being both rich in charged amino acids [14], we suggest that the negatively charged EP molecule binds to the positively charged tubulin/actin binding

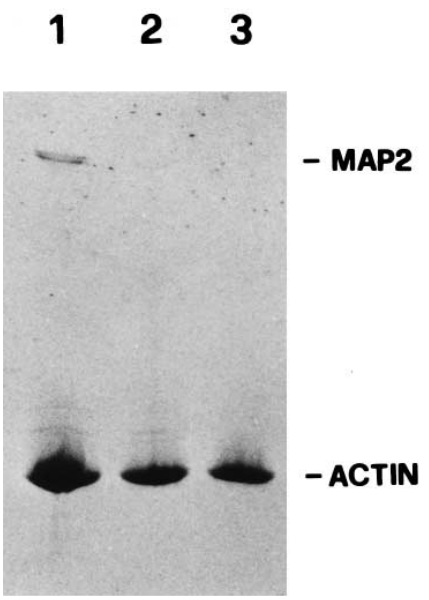

Fig. 4. Effect of EP on actin crosslinking. F-Actin was incubated either with MAP2 (lane 1) or with MAP2 in the presence of 180 μm EP (lane 2) or in the absence of MAP2 (lane 3) and sedimented by centrifugation through a sucrose cushion. The pellets were analysed by SDS-PAGE, the positions of MAP2 and actin are indicated.

domain of MAP2 producing its effect both on microtubules and actin filaments. This hypothesis is supported by the observation that under our experimental conditions estramustine does not affect MAP2:actin interaction, even at high concentrations, and that the effect of estramustine derivatives on microtubule assembly depends on the charge of the substituent [7].

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